REMARKS

Claims 1-36 are pending in the present application.

The Examiner has required election in the present application among:

Group I, claims 1, 3, and 4-21, drawn to an isolated polypeptide and a method of detecting anti-leishmanial antibodies by providing a solid support with a polypeptide bound to it and a diagnostic kit for detecting anti-leishmanial antibodies comprising a polypeptide as claimed in claim 1, an anti-human secondary antibody or a protein, wherein said anti-human secondary antibody or the protein is conjugated to an enzyme or a label, and conventional reagents for detecting said antibodies;

Group II, claim 2, drawn to an isolated polynucleotide;

Group III, claims 22 and 23, drawn to a method of making antibodies;

Group IV, claims 24-31, drawn to a method of detecting Leishmanial antigens in a sample using antibodies;

Group V, claims 32-36, drawn to a diagnostic kit for detecting Leishmanial antigens comprising antibody bound to a solid support or carrier, antibody conjugated to an enzyme or a label and conventional reagents for detecting Leishmanial antigens;

For the purpose of examination of the present application, Applicants elect, with traverse, Group I, Claims 1, 3, and 4-21. The species of SEQ ID NO: 6 is elected for initial prosecution.

All of claims 1 and 3-21 "read on" the elected species.

Traverse of the Restriction Requirement is as follows.

The present application is a PCT application and as such Unity of Invention under Rules 13.1 and 13.2 governs restriction practice at the national stage. Applicants submit that the Examiner has not considered that the different groups of invention set forth are all joined by having the common special technical feature of relating to the novel and inventive polypeptide of the present invention. That is, peptides and nucleic acids of the invention are useful in making

the antibody of the invention, and the kits and methods for detection of Leishmanial antigens relate to uses of the polypeptide per se to detect antibodies in samples.

Attached as Exhibit 1 is a copy of a recent publication (Sivakumar et al., *Infection, Genetics and Evolution* 8:313 (2008)) showing that the polypeptide of the presently elected invention demonstrates unexpectedly improved sensitivity and lack of cross-reactivity with irrelevant antibodies in diagnostic assays.

In view of the above, at least Groups II and III should be rejoined for prosecution in the present application.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Mark J. Nuell, Ph.D., Registration No. 36,623, at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to our Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under § 1.17; particularly, extension of time fees.

Dated: April 16, 2009

Respectfully submitted,

By _____

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Expression and characterization of a recombinant kinesin antigen from an old Indian strain (DD8) of *Leishmania donovani* and comparing it with a commercially available antigen from a newly isolated (KE16) strain of *L. donovani*

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Abstract

Recently we had prepared a recombinant antigen (Ld-rKE16) from a newly isolated Indian strain of *Leishmania donovani* (MHOM/IN/KE16/1998) with high sensitivity and specificity and the same has been commercialized. While comparing the sequence data of kinesin gene of this (KE16) strain and its expressed protein with another commercially available recombinant antigen (Lc-rK39) from kinesin gene of *L. chagasi* we found significant genetic and amino acid variations. This prompted us to undertake the present study to unravel whether the kinesin gene and its expressed protein from another old but Indian isolate of *L. donovani* (MHOM/IN/DD8/1968) had any genetic and amino acid heterogeneity. Sequencing of the kinesin gene revealed that the kinesin gene of DD8 strain is 3016 bp long and has immunodominant region consisting of 4.8 tandem repeats, 117 base pairs each. Further blast analysis of the immunodominant regions of 5 strains of *L. donovani* revealed that it has only 79% homology with *L. chagasi,* and 80% homology with *L. infantum*; while it had 82% homology with Sudan strain of *L. donovani*, 82% with another (Morena) strain of Indian *L. donovani* but highest homology of 83% with *L. donovani KE16* strain of India. We also evaluated the diagnostic potential of the recombinant DD8 antigen (Ld-rDD8) and compared the results with that of Ld-rKE16. The study revealed that Ld-rKDD8 antigen was less sensitive and specific as compared to rKE16 antigen for the diagnosis of visceral and post-kala-azar dermal leishmaniasis. This was probably due to prolong *in vitro* culture maintenance of the DD8 strain.

Keywords: Kala-azar; *Leishmania donovani*; Kinesin; Ld-rKE16; Ld-rDD8; Immunodominant region

1. Introduction

Leishmaniasis, a vector-borne disease caused by an obligate intramacrophage protozoan, is characterized by diversity and complexity. The parasite is transmitted from infected person to uninfected person through the bites of a tiny *phlebotomine* sandfly. There are 500 species of *phlebotomine* sandflies, of these only 30 species are suspected or proven vectors of this parasite (Herwaldt, 1999; Bora, 1999; Desjeux, 2001; Singh and Sivakumar, 2003; Singh, 2006). In India *Phlebotomous argentipes* is the most common sandfly vector.

Human leishmaniasis presents in four different forms with a broad range of clinical manifestations but visceral leishmaniasis (VL), also known as kala-azar (KA), is the most severe form of the disease. If untreated, it has a mortality rate of almost 100%. It is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia; and is caused by the species of *Leishmania donovani* complex that consists mainly of *L. (d) infantum, L. (d) donovani & L. (d) chagasi* (Herwaldt, 1999; Desjeux, 2001; Singh, 2006).

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Characteristically, in acute cases of visceral leishmaniasis a marked elevation of specific and non-specific immunoglobulin G (IgG), IgM and IgA takes place due to polyclonal activation of the B cells. Taking advantage of this phenomenon, several serological methods have been developed for the diagnosis of kala-azar. However, the specificity of these tests is dependent upon the antigen or epitopes used in the test systems and vary in sensitivity and specificity (Singh and Sivakumar, 2003; Singh, 2006).

Recently, recombinant DNA technology has revolutionized the diagnostic medicine, and several recombinant antigens are reported useful in the diagnosis of visceral Leishmaniasis. However, only a few have shown promise while others lacked sensitivity or specificity (Singh and Sivakumar, 2003; Singh, 2006). The first most promising recombinant antigen was expressed from the kinesin related gene of *Leishmania chagasi*, which encodes this protein with repetitive epitopes of 39 amino acid residues (K39). This recombinant antigen (Lc-rK39) has been used extensively for the sensitive and specific diagnosis of VL caused by members of the *L. donovani* complex (Burns et al., 1993; Qu et al., 1994; Singh et al., 1995a,b; Badaro et al., 1996; Maalej et al., 2003). However lately, the same antigen was reported to be less sensitive and specific in some parts of the world (Ozensoy et al., 1998; Jelinek et al., 1999; Zijlstra et al., 2001).

The reasons for the varied sensitivity and specificity of the rK39 in parts of the world are not fully known, but it was strongly associated with geographical distribution of causative agent. Though the kinesin antigen gene is reported to be conserved in all visceralising species, there could be a significant sequence and antigenic variation in different members of *L. donovani* complex causing the disease in different geographical regions (Zijlstra et al., 2001; Beverley et al., 1987; Singh et al., 1995a,b; Mauricio et al., 1999; Sundar and Rai, 2002; Dey and Singh, 2007). Since, the whole kinesin gene from *Leishmania donovani* has not yet been characterized, we have carried out the molecular characterization of kinesin gene from two Indian isolates of *L. donovani* MHOM/IN/KE16/1998 and *L. donovani* MHOM/IN/DD8/1968. The antigen cloned and characterized from a recently isolated KE16 strain of *L. donovani* (MHOM/IN/KE16/1998) has already been published elsewhere (Sivakumar et al., 2006). This strain will be referred as KE16 in short, throughout the manuscript for convenience. In this study we report the genetic heterogeneity in the kinesin gene from a 35-year-old laboratory maintained strain and compare it with a new clinical isolate. Further the expressed recombinant kinesin protein from this old laboratory maintained strain is characterized and evaluated for its diagnostic potential.

2. Materials and methods

2.1. Parasite

A WHO standard strain of *Leishmania donovani* (MHOM/IN/DD8/1968) is being maintained in Medium 199 (Hi-Media®, India) supplemented with 10% heat inactivated fetal calf serum (FCS) at 25 °C in a BOD incubator in our laboratory for the last 10 years (Singh et al., 1995a,b). The strain was originally isolated from a kala-azar patient of Bihar, India in 1968. This strain will be referred as DD8 in short, throughout the manuscript for convenience.

2.2. PCR cloning and sequencing of the kinesin gene from L. donovani

The nuclear DNA (nDNA) from the promastigotes was isolated as described previously (Sivakumar et al., 2006). The kinesin antigen-coding region was amplified from *L. donovani* nDNA. Based on the sequence of the *L. chagasi* kinesin gene [GenBank Accession L07879] and *L. donovani* (KE16) five oligonucleotide primers were designed to obtain three overlapping fragments of the target gene by PCR amplification (Burns et al., 1993; Sivakumar et al., 2006). The reason for using five sets of primers is to overcome the problem of amplification and cloning of large 3016 bp fragment. Fig. 1 shows the codon position (in parenthesis) and direction of the various primers used in the study. The primer sets LKF1 (93): 5′ CGGCGCGTCGGTGTCTTTGAT 3′ and LKR1 (1803): 5′ AGGTCCGCCGCACGCTTCTG 3′ were designed to amplify a product of 1711 bp size spanning the region between 93 and 1803 bp from the already published Ld-KE16 gene sequence. The primer sets LKF 2 (1527): 5′ GCGGGAACTCGAA-GACGTTCAT 3′ and LKR2 (2679): 5′ CGTGGCCCT-CGTGTTCTCGC 3′ were designed to amplify the upstream sequence spanning between 1527 and 2679 bp in the Leishmania kinesin gene. This region included the conserved non-repeat sequence and the 117 bp tandems repeat region unit of kinesin immunodominant domain. The primer set LKF3 (2452): 5′ GAGCAGCAGCTTCGTGACTCC 3′ and LKR2 (3016): 5′ CGTGGCCCTCGTGTTCTCGC 3′ were designed to amplify the immuno-dominant tandem repeats region of strain DD8.

PCR amplified sequences were TA cloned into pGEM-TE vector (Promega®, USA). At least three positive clones for each PCR product were sequenced to ensure fidelity (Microsynth Inc®, Switzerland) using universal primers. Partial sequencing results were obtained for the terminal ends of 1711 bp insert in pGEM-TE clone of LKF1/LKR1 primer set-amplified product. To obtain a full length sequence, two

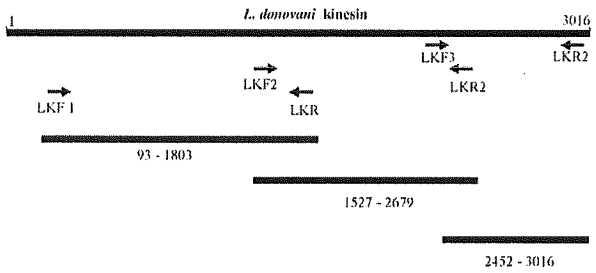

Fig. 1. Strategy used in cloning and sequencing of kinesin gene of Indian strains of *Leishmania donovani*. Arrow denotes the primer positions. Overlapping products generated by PCR and cloned in pGEM-T were shown in thick lines.

internal fragments; one spanning from nucleotides positions of 99–769 bp obtained by digestion with EcoRI/KpnI and the second spanning from nucleotides positions of 765–1676 bp obtained by digestion with BamHI/KpnI, were subcloned individually in pUC18 and sequenced using M13 primers (Lu et al., 1994).

The sequences were assembled to form a contig using Seqman II® (Lasergene package) and sequence analyses were performed using Lasergene: Edit, Megalign, etc (DNA star® Inc.). For multiple sequence alignment, the poorly aligned positions and divergent regions of a DNA or protein alignment were eliminated using Gblocks server (version: Gblocks 0.91b) preserving the default parameters. The Gblocks conserved blocks were then taken for Clustal W multiple sequence alignment. The homology of the sequences was searched using NCBI BLAST option (Altschul et al., 1997).

2.3. B-cell epitope prediction

The immunodominant amino acid repeat sequences from *L. donovani* DD8 strain were subjected to B-cell epitope prediction using BcePred prediction server [www.imtech.res.in/raghava/bcepred/]. This server predicts B cell epitope regions in an antigen sequence, using physicochemical properties.

2.4. Expression and purification of Ld-kinesin

After validating the sequences of PCR product the relevant fragments were sub-cloned in the *E. coli* expression vector with 6XHis tag. A 564 bp PCR product of LKF3/LKR2 primers was obtained from the relevant pGEM-TE clones by digestion with PstI and NcoI restriction sites present in the vector flanking the insert. The fragment was gel purified and ligated in to the appropriate restriction sites of the pRSET-C vector (Invitrogen®) in frame. The ligation mixture was then transformed to *E. coli* BL21 (PLysis) cells following standard protocol (Sambrook et al., 1989). The induction of expression of recombinant protein was performed according to the method described earlier (Sivakumar et al., 2006).

The 6XHis tagged fusion protein was recovered in the soluble fraction and purified by immobilized-metal affinity chromatography (IMAC) using Ni-NTA (QIAGEN) following manufacturer's protocol.

2.5. Immunoblot analysis

2.5.1. Immunoblot using patient and control sera

The purified protein was separated on 12% SDS-PAGE and transferred onto nitrocellulose membranes using a semi-dry blotting apparatus (Bio-Rad,® USA). Unbound membrane was blocked with PBS containing 5% non-fat dried milk and probed with sera from VL and PKDL patients, healthy controls, negative controls (1:200 dilutions in PBS containing 5% non-fat dried milk) were also included. Alkaline Phosphatase conjugated Anti-human IgG (Boehringer Mannheim Gmbh, Germany) was used as the secondary antibody (1:4000 dilution). After several washes with phosphate buffered saline containing 0.02% Tween-20 (PBS-T), the membrane was developed by addition of BCIP-NBT (Amresco®, USA). After SDS-PAGE and semi-dry transfer of proteins to the nitrocellulose membranes, detection with Penta-anti-His Antibody-HRP conjugates (QIAGEN, Germany) was done by chromogenic method as described earlier (Sivakumar et al., 2006).

2.5.2. Dot blot

The sensitivity of the purified antigen was tested by dot blot with increasing concentration of antigen viz., 25, 50, 100, 150 and 200 ng and probed with kala-azar patient sera (1:200 dilutions) and endemic healthy control sera (1:200 dilutions). The purified protein was directly spotted onto the nitrocellulose membrane, air dried and Western blotted with patient sera as above.

2.5.3. Patient sera

Sera samples from 469 individuals were used in this study. This included 150 well-characterized samples from patients of acute VL (confirmed serologically and parasitologically) and 7 patients of PKDL, from Bihar, India. The control group consisted of 50 healthy individuals from the same endemic area Bihar (India) and 80 from non-endemic area (Delhi, India). There were 182 other disease controls including microbiologically confirmed cases of pulmonary tuberculosis (92), asymptomatic anti-HIV positive (32), HBsAg positive (27), and anti-HCV antibody positive (31) cases. The sera from these patients were included to check the cross reactivity and specificity of our antigen in different infections. All Indian sera were collected adopting standard protocols and institutional guidelines.

2.6. Enzyme-linked immunosorbent assay

2.6.1. Recombinant antigen coating and ELISA on clinical samples

ELISA using the purified recombinant antigen was first standardized by using different concentrations of antigen following the checkerboard method. The optimal concentration of the antigens was determined to be 50 ng/well and 1:100 serum dilutions (Sivakumar et al., 2006). Same quantity of rKE16 was also used to coat other plates for parallel ELISA tests. The ELISA was performed using known kala-azar patient and other controls as described below. Briefly, the purified recombinant antigens were diluted in coating buffer (0.1 M bicarbonate buffer, pH 9.2), and 1 polystyrene microtitre plate was sensitized overnight at 4 °C with (50 ng/well) recombinant antigen prepared either from DD8 strain of *L. donovani* (Ld-rKE29) and KE16 strain of *L. donovani* (Ld-rKE16) followed by blocking with 200 μl of 1% bovine serum albumin (Sigma Chemical Co., St. Louis, USA) for 1 h. The plate was washed 3 times with phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T), for 1 h at room temperature. After four washes with PBS, 50 μl serum diluted (1:100) in PBS-T was added to each well and incubated for 2 h at room temperature. The wells were washed again and incubated with 50 μl of goat

anti-human IgG conjugated (Boehringer Mannheim Gmbh, Germany) with alkaline phosphatase at 1:1000 dilution for another 2 h at room temperature, followed by washing. To this, 50 μl of *p*-nitrophenylphosphate in diethylamine buffer was added, incubated for 30 min at 37 °C and the reaction was stopped with 50 μl of 3N H_2SO_4. The optical density of each well was measured at 450 nm in an ELISA plate reader. The value of mean optical density (OD) plus two standard deviations of OD values of non-endemic healthy controls was taken as cutoff for evaluating both the antigens in parallel.

2.7. Statistical analysis

The OD values from the automated ELISA reader were directly transferred to Microsoft Excel, the mean and standard deviation were calculated using Excel and the graphs were plotted using appropriate software.

3. Results

3.1. Cloning of kinesin gene

PCR amplification of DD8 strain of *L. donovani* was carried out using the primers LKF1, LKR1, LKF2, LKR2 and LKF3 in combinations as shown in Section 2. A 1711 bp product was obtained from the strain following PCR amplification using the primer LKF1 and LKR1 spanning the region between 93 and 1803 in the Leishmania kinesin gene (Fig. 2A). Another set of primers LKF2 and LKR2 yielded a single 1154 bp product upon PCR amplification spanning the region between 1527 and 2679 in the Leishmania kinesin gene (Fig. 2B). This region also includes one 117 bp repeat unit of kinesin immunodominant domain and the third set targeting the conserved immunodominant repeat using primers LKF3 and LKR2 yielded multiple bands with sizes from approximately 117, 234, 351 and 564 (Fig. 2C). We eluted the strongest band of 564 bp from the gel closest in size to the 468 bp long tandem repeat region of *L. donovani* (KE16) and used it for cloning in pGEM-TE vector (Sivakumar et al., 2006).

3.2. Sequencing of kinesin gene

Complete nucleotide sequence of kinesin gene obtained from the strain DD8 was found to be 3016 bp long comprising one long open reading frame (ORF) of 2670 bp (GenBank No. AY615887). The immunodominant repeat domain started from the nucleotide at the position 2453 bp and extended till the last nucleotide at the position 3016 bp spanning a region of 564 bp corresponding with 4.8 units of 117 bp tandem repeats. The deduced amino acid sequence revealed that the open reading frame encodes 890 amino acids with a predicted molecular mass of 98.7 kDa and p*I* of 5.79 with a charge of (−) 12.03. The deduced amino acid sequence of the immunodominant repeat revealed that it encodes 188 amino acids with a predicted molecular mass of 20.7 kDa and p*I* of 5.31 with a net charge of (−) 4.01 at pH 7.0.

3.3. Sequence analysis and B-cell epitope prediction

BLAST analysis indicated that DD8 kinesin gene ORF is 89% identical to that of *L. chagasi* and *L. infantum* gene coding for kinesin related protein and 90% identical to that of *Leishmania donovani* KE16 strain, sequence. Further, specific analysis of the immunodominant regions revealed that these regions have more variations as compared to total kinesin ORF as whole. Analysis revealed that *Leishmania donovani* DD-8 immunodominat region has 82%, homology with Sudan strain of *L. donovani* and 82% & 83% homology with two isolated

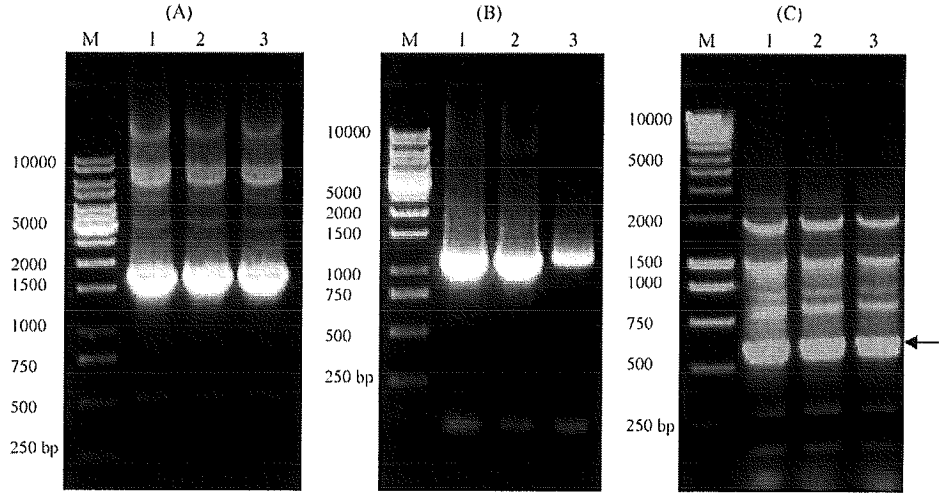

Fig. 2. (A) PCR amplification of 1711 bp partial kinesin gene using the primer set LKF1/LKR1; Lane M: 1 kb DNA molecular weight marker (MBI Fermentas), Lane 1–3: Shows amplification of 1711 bp product. (B) PCR amplification of 1154 bp partial kinesin gene using the primer set LKF2/LKR2; Lane M: 1 kb DNA molecular weight marker (MBI Fermentas), Lane 1–3: shows the amplification of 1154 bp product. (C) PCR amplification of 564 bp Immunodominant 117 bp tandem repeat epitope of kinesin gene from the strain DD8 using the primer set LKF3/LKR2; Lane M: 1 kb DNA molecular weight marker (MBI Fermentas), Lane 1–3: shows the amplification of 564 bp product.

Table 1
Multiple sequence alignment of immunodominant domain (IMM) at nucleotide levels of 5 strains of Leishmania

L. donovani (MHOM/IN/DD8)	1	GTT GAG CAG CAG CTT CGT GAC TCC GAG ACG CGC GCT GCG GAG CTG AAA GCC GAG CTG GAG	60
L. donovani (MHOM/SD)	1	--- --- --- --- --- --C --A --- --- G-- --- --- -G- --- --- CG- --- --- --- ---	60
L. donovani (MHOM/IN/KE16)	1	-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --T --G --- T-A ---	60
L. donovani (MHOM/IN/Morena)	1		60
L.infantum (JPCM5)	1	--- --- --- --- --- --- --- --- --- --- --- --C --- --- --- CG- --- --- --- ---	60
L. chagasi (BA-2)	1	--- --- --- --- --C --A --- --- G-- --- --- --- --- --- CG- --- --- --- ---	60
L. donovani (MHOM/IN/DD8)	61	GCC ACT GCT GCT GCG AAG ACG TCG GTG GAG CAG GAG CGT GAG AAG ACG AGG ACG GCT CTG	120
L. donovani (MHOM/SD)	61	--- --- --- --- --- --- T-- --- -C- --- --- --C --C --- --C --- --- G-T --- ---	120
L. donovani (MHOM/IN/KE16)	61	--G --- --- --- --- --- T-- --- -C- --- --- --C --C --- --C --- --- --- --G ---	120
L. donovani (HM/IN/Morena)	61		120
L. infantum (JPCM5)	61	--- --- --- --- --- --- T-- --- -C- --- --- --C --C --- --- --- T-- --- --- ---	120
L. chagasi (BA-2)	61	--- --- --- --- --- --- -T- --- -C- --- --- --- --A --- -G- --- --- --A --- ---	120
L. donovani (MHOM/IN/DD8)	121	GAG GGG CGC GCT GCG GAG CTG GCT CGC AAA CTG GAG GCG ACT GCT TCT GCG AAG AAT TTG	180
L. donovani (MHOM/SD)	121	C-- -C- --- --- --- --- --- -AA G-- G-G --- --- --C --- --- G-- --- --- -CG -C-	180
L. donovani (MHOM/IN/KE16)	121	--- --- -A- --- --- --- --- -AA G-- C-G --- --- T-C --- --- G-- --- --- -CG -C-	180
L. donovani (MHOM/IN/Morena)	121	GAG -A- -A- --- --- --- --- -AA G-- C-G --- --- T-C --- --- G-- --- --- --- -C-	180
L. infantum (JPCM5)	121	--- -A- --- --- --- --- --- --G A-- C-G --- --- T-T --- A-- G-- --- --- --- -CA	180
L. chagasi (BA-2)	121	--- -A- --- --- --- --- --- --G A-- C-G --- --- T-C --- A-- G-- --- --- --- -CA	180
L. donovani (MHOM/IN/DD8)	181	GTA GAG CAG GAC CGC GAG AGG ACG AGG GCC ACC TTG GAG GAA CGA CTT CGT ATT GCT GAG	240
L. donovani (MHOM/SD)	181	-CG --- --- --- --- --- --- -T- --- -T- --G --- --- --G --C --- --C G-C --- ---	240
L. donovani (MHOM/IN/KE16)	181	-CG --- --- --- --- --- -AC --- --- --- G-G --- --- C-G --G --- --G -A- --C ---	240
L. donovani (MHOM/IN/Morena)	181	-CG --- --- --- --- --- -AC --- --- --- G-G --- --- C-G --- T-- --G -A- --C ---	240
L. infantum (JPCM5)	181	--G --- --- --- --- --- -AC --- --- --- --- --A --- C-G --- --- --- -C- --C ---	240
L. chagasi (BA-2)	181	-CG --- --- --- --- --- --C --- --- --- --- --A --- C-G --- --- --- -C- --C ---	240
L. donovani (MHOM/IN/DD8)	241	GTG CGC GCT GCG GAG CTG GCA GGA GTG CTG GAG GCC ACT GCT GCT GCG AAG ACG GCG GTG	300
L. donovani (MHOM/SD)	241	T-- --- --- --- --- --- A-G --- --- --- --- --- --- --- --- --- --- --- --- T-- -C-	300
L. donovani (MHOM/IN/KE16)	241	-A- --- --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- T-- T-- -C-	300
L. donovani	241	-A- -A- --- --- --- --- -A- --C CA- --- --- T-- --- --- --- --- --- --- T-- -C-	300

Table 1 (*Continued*)

L. infantum (JPCM5)	241	-A- --- --- --- --- --- --- --- -A- --- --- T-- --- --- --- --- --- T-- T-- -C-	300
L. chagasi (BA-2)	241	-A- --- --- --- --- --- --- --- -A- --- --- --- --- A-- --- --- --- -T- T-A -C-	300
L. donovani (MHOM/IN/DD8)	301	GAG CAG GAG CGT GAG AGG ACG AGG GCC GCC TTG GAG CAG CAG CTC CGC GAA TCC GAG GCG	360
L. donovani (MHOM/SD)	301	--- --- --T --- --- --- --- --- A-- A-G --- C-- G-- --- --T --- --- --- --- ---	360
L. donovani (MHOM/IN/KE16)	301	--- --- --C --C --- -AC --- --- --- A-- --A --- --- --- --T --- --- --- --- ---	360
L. donovani (MHOM/IN/Morena)	301	--- --- --C --C --- -AC --- --- --- --G --- --- --- --- --T --- --- --- --- -A-	360
L. infantum (JPCM5)	301	--- --- --C --C --- -AC --- --- --- --G --- --- --- --- --T --T --C --- --- -A-	360
L. chagasi (BA-2)	301	--- --- --C --C --- --C --- --- --- A-- --A --- --- --- --T --- --- --- --- -A-	360
L. donovani (MHOM/IN/DD8)	361	CGC GCT GCG GAG CTG GCT GCG CAG CTG GAA GCC GCT GCT GCG GCG AAG ACG TCG GTG GAG	420
L. donovani (MHOM/SD)	361	--- --- --- --- --- AAG --- --- --- --G --- A-- --- --T --- --- T-- --- -C- ---	420
L. donovani (MHOM/IN/KE16)	361	--- --- --- --- --- -A- --- --- --- --- --- A-- --- --T --- --- T-- --- -C- ---	420
L. donovani (MHOM/IN/Morena)	361	--- --- --- --- --- -A- --- A-- T-A --- -G- --- --- --T --- --- T-- --- -C- ---	420
L. infantum (JPCM5)	361	--- --- --- --- --- --- -A- --- --- --- --- A-- --- --T --- --- T-- --- -C- ---	420
L. chagasi (BA-2)	361	--- --- --- --- --- --- -A- --- --- --- --- A-- A-- --T --- --- -T- --A -C- ---	420
L. donovani (MHOM/IN/DD8)	421	CAG GAG CGT GAG AAC ACG AGG GCC ACC TTG GAG GAG CGG TTG CGG CTC GCT GAG GTC CGC	480
L. donovani (MHOM/SD)	421	--- --C --C --- --- --- --- --T G-- --- --- --- AA- C-- G-- -A- --- --- --- ---	480
L. donovani (MHOM/IN/KE16)	421	--- --C --C --- --- --- --- --- G-G --- --- C-- --- --- --A A-- --- --- -AG ---	480
L. donovani (MHOM/IN/Morena)	421	--- --C --C --- --- --- --- --- G-G --- --- C-- --C --- --A A-- --- --- -AG -A-	480
L. infantum (JPCM5)	421	--- --- --C --- --- --- --- --- G-G --- --- C-- --C --- --- --- --- --- -AG ---	480
L. chagasi (BA-2)	421	--- --C --C --- -G- --- --- --- --- --A --- C-- --C --- --- --- --- --- -AG ---	480
L. donovani (MHOM/IN/DD8)	481	GCT GCG GAG CTG GCA GCG CGG CTA AAG AGC ACT GCT GCT GTT AAG TCC GCG ATG GAG CAG	540
L. donovani (MHOM/SD)	481	--- --- --- --- --- --C --G --- --- --- --- --- --- -AC --- G-A T-- --C --- ---	540
L. donovani (MHOM/IN/KE16)	481	--- --- --- --- --- --- -A- --G G-- --- --- --- --- -CG --- --- --- GC- --- ---	540
L. donovani (MHOM/IN/Morena)	481	--- --- --- --- -A- --C -A- --G G-- TC- --- --- --- -CG --- -A- --- GC- --- ---	540
L. infantum (JPCM5)	481	--C --- --- --- --- --- -A- --G G-- --- --- --- --- -GA --- --A --- --- --- ---	540
L. chagasi (BA-2)	481	--- --- --- --- --- --- -A- --G G-- --- --- --- --- -CG --- --- --- GC- --- ---	540
L. donovani (MHOM/IN/DD8)	541	GAC CGC GAG AAC ACG AGG GCC ACG	564
L. donovani (MHOM/SD)	541	--G A-G --- -G- T-- --- ---	564
L. donovani (MHOM/IN/KE16)	541	--- --- --- --- --- --- --- ---	564
L. donovani (MHOM/IN/Morena)	541	--- --- --- --- --- --- --- ---	564
L. infantum (JPCM5)	541	--- --- --- --- --- --- --- ---	564
L. chagasi (BA-2)	541	--- --- --- --- --- --- --- ---	564

Indian strains of *L. donovani* (Morena) and *L. donovani (KE16)*, respectively (Gerald et al., 2007; Sivakumar et al., 2006; Dey et al., 2007). However, it had 79% and 80% homology with *L. chagasi* and *L. infantum*, respectively (Burns et al., 1993). The multiple sequence alignment of kinesin gene immunodominant at nucleotide level is presented in Table 1.

The NCBI data bank searches revealed the presence of kinesin motor catalytic domain in the N-terminal of the kinesin amino acid sequence, spanning initial 530 amino acid regions. Sequence similarity on an average upto 40.5% between DD8 kinesin motor domain and members of the superfamily of kinesin-related proteins of *Caenorhabditis elegans, Homo sapiens, Mus musculus, Rattus norvegicus, Drosophila melanogaster, Cochliobolus heterostrophus, Botryotinia fuckeliana*, was found in this region.

Potential epitope-containing peptide was detected in the recombinant antigen and is presented in Table 1. The number of such antigenic regions detected varied from 6 to 11 among the strain of *L. donovani*, and *L. chagasi* with a length of 5–9 amino acids as shown in Table 2. A total of 6 epitopes were found in the immunodominant region spanning a length of 188 amino acids in the DD8 strain of *L. donovani*.

3.4. Large scale expression and purification

The recombinant proteins were purified using Immobilized-metal affinity chromatography (IMAC) using Nitrilotriacetic acid. The recombinant proteins were optimally eluted at 160 mM Imidazole concentration. We obtained an expression level of 5 mg of purified recombinant protein from 1 l culture. The purified recombinant protein from the strain DD8 migrated at a predicted molecular weight of ~29 kDa containing an 8.3 kDa of plasmid fusion protein. Protein composition analysis reveals that the 29 kDa protein contains 38 acidic (D & E) and 34 basic (K & R) amino acid residues. The expressed protein product was named as Ld-rDD8 and the sensitivity and specificity of this protein was studied.

Table 2
Sequences identified as B-cell epitopes in the kinesin immunodominant domain from *L. donovani*, *L chagasi* by Bcepred server

DD8IMM	KE16IMM	LCIMM
EQEREKT	LRDSEERA	LRESEERS
EQDRERT	LRESEEHAA	AEQDRENT
EQERERT	AQDRENT	LRDSEERA
QERENT	AEQDRENT	LRDSEERA
LRESE	AEQDRENT	LRDSEERA
DRENT	RLRESEERA	LRDSEERA
	AEQDREN	LRDSEERA
		AEQDREN
		EQDRES
		EQDRES
		EQDRES

DD8IMM: Immunodominant domain from the strain DD8 of *L. donovani*; KE16 IMM: Immunodominant domain from the strain KE16 of *L. donovani*, LCIMM: Immunodominant domain from the species *L. chagasi*.

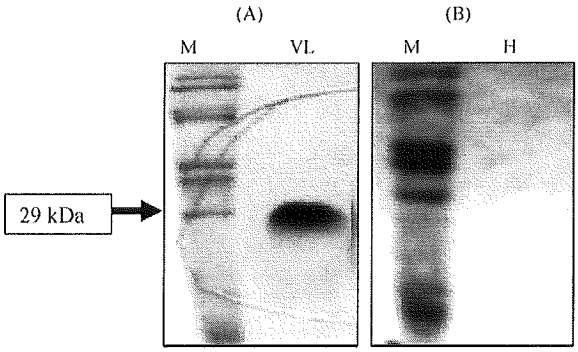

Fig. 3. (A) Western blot analysis with VL positive patient sera for the purified protein; Lane M: Pre-stained Molecular weight marker; Lane VL: Purified protein from DD8 (~29 kDa) give strong signal with VL positive sera. (B) Western blot analysis with pooled Healthy endemic control sera for the purified protein; Lane M: Pre-stained Molecular weight marker, Lane H: No cross-reactivity of antibodies from healthy sera with DD8 purified protein.

3.5. Evaluation of the sensitivity and specificity of recombinant antigens Ld-rDD8 against patient antibody serum

The purified antigens were first run on SDS-PAGE and then subjected to immunoblotting with penta-anti-his HRP conjugate antibody. The recombinant protein showed strongly positive results with VL sera and negative results with control sera indicating that the recombinant antigens from Indian strain of *L. donovani* were specific (Fig. 3A and B). The sensitivity of the purified antigen were tested by dot blot with increasing concentration of antigens viz., 25, 50, 100, 150 and 200 ng and probed with kala-azar patient serum (1:200 dilution) and healthy endemic control serum. The sensitivity and specificity of the antigen was compared with that of rKE16 antigen. The results show that as less as 25 ng of the antigen could detect the antibodies from the sera (Fig. 4A). However the analytical sensitivity of the antigen was less than that of rKE16 antigen (Fig. 4B). Nevertheless, both the antigens showed low or no cross reactivity with endemic healthy control sera in the dot blot suggesting their specificity (Fig. 4A and B).

The prevalence of *L. donovani* recombinant kinesin specific antibodies in the sera from the population of VL patients to the protein was assayed by ELISA against a panel of 157 parasitologically confirmed (150 VL and 7 PKDL), 50 Endemic, 80 non-endemic healthy control, 92 patients of Tuberculosis, 32 HIV positive patients, 31 HCV infected and 27 Hepatitis B infected patient sera. The optimal concentration of the antigen was determined to be 50 ng/well and the sera dilution was 1:100. The results showed that all the 150 (100%) VL and 7 PKDL patients sera were recognized by both the antigens (Table 3) with mean OD values of 1.26 ± 0.56 for Ld-rDD8 and 1.42 ± 0.57 for rKE16, indicating that analytical sensitivity of Ld-rDD8 was slightly poor. Six of the samples from endemic control group were also found to be false positive by Ld-rDD8, whereas none of the control group showed positive results with rKE16 (Table 3). This showed that the antigen was not good for ELISA or rapid test formats.

Table 3
Comparative sensitivity and specificity of recombinant antigens prepared from DD8 and KE16 strains of Indian *L. donovani*

Sample type	Ld Ld-rDD8 antigen		Ld rKE16 antigen	
	Sample tested	Sample + ve	Sample tested	Sample + ve
Sera from parasitologically confirmed VL cases	150	150 (100%)	150	150 (100%)
Sera from parasitologically confirmed PKDL cases	7	7 (100%)	7	7 (100%)
Endemic control sera	50	6 (12%)	50	0
Non-endemic control sera	80	0	80	0
Tuberculosis patient sera	92	0	92	0
HIV positive patient sera	32	0	32	0
HCV positive patient sera	31	0	31	0
HBsAg positive patient sera	27	0	27	0

4. Discussion

Present work is continuation of our efforts to identify a novel kinesin related antigen from Indian strains of *L. donovani*. Recently we reported cloning and sequencing of a novel recombinant antigen from MHOM/IN/KE16/1998 strains of *L. donovani* (Sivakumar et al., 2006). Here we report cloning and sequencing of its homologous gene of *Leishmania donovani* MHOM/IN/DD8/1968, a laboratory maintained strain for the last 39 years. After preparing a recombinant antigen successfully from a recent clinical isolate KE16, we wanted to see if the *Leishmania donovani* DD8 strains, maintained *in vitro* and has undergone numerous subcultures, has developed some genetic variation. This study also evaluates the sensitivity and specificity of this recombinant antigen for the serodiagnosis of VL and PKDL vis-à-vis a recombinant antigen (Ld-rKE16) prepared from another Indian strain (MHOM/IN/KE16/1998) of *L. donovani* isolated from a human recently (Sivakumar et al., 2006).

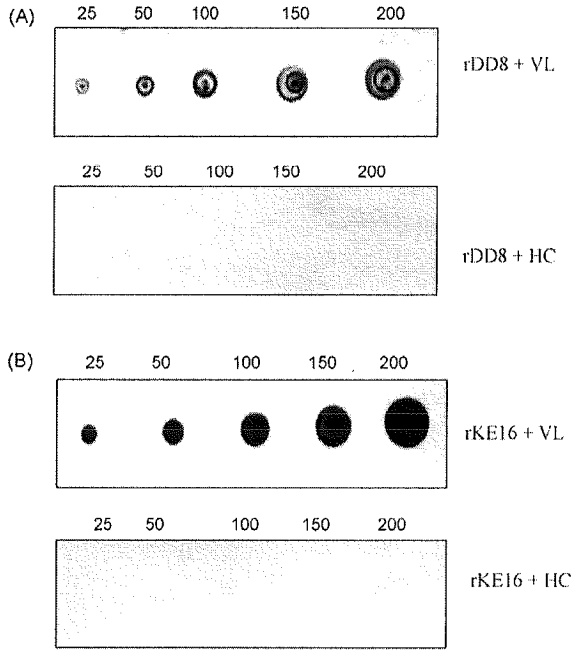

Fig. 4. (A) The sensitivity of the purified antigen from DD8 tested by dot blot with increasing concentration of antigens viz., 25, 50, 100, 150 and 200 ng and probed with kala-azar (VL) patient serum and endemic healthy control (HC) serum. (B) The sensitivity of the purified antigen from rKE16 tested by dot blot with increasing concentration of antigens viz., 25, 50, 100, 150 and 200 ng and probed with kala-azar (VL) patient serum and endemic healthy control (HC) serum.

The BLAST search confirmed the sequences obtained were of kinesin immunodominant domain and contained 4.8 tandem repeats (117 bp each) as compared to 4 tandem repeats in KE16 strain (Sivakumar et al., 2006). The kinesin ORF of DD8 strain encodes 890 amino acids, containing 4.8 units of 39 amino acid tandem repeats at the c-terminal immunodominant repeat domain. The obtained gene sequence was compared with 5 other *Leishmania donovani* complex strains (Table 1). These strains included 3 *L.(d) donovani* strains (MHOM/IN/KE16/1998, MHOM/IN/Morena/2004, *MHOM/SD/DQ831678*), one *L.(d) infantum* (JPCM5) and one *L.(d) chagasi* (BA-2). The BLAST analysis and comparison with other 5 strains showed that the structural organization of the kinesin gene is conserved between *L.(d) donovani*, *L.(d) infantum* and *L.(d) chagasi* at kinesin motor domain region. However there is much variation at immunodominant region. The BLAST results showed that as expected the DD8 kinesin immunodominant domain was more closely related to *L.(d) donovani* KE16, Morena and Sudan strains (Gerald et al., 2007; Sivakumar et al., 2006; Dey et al., 2007) than *L. (d) chagasi or L.(d) infantum* (Burns et al., 1993). Present study also showed that statement of Burns et al. (1993) that kinesin region is conserved in all visceralizing species is only partially correct. While all the 6 strains showed presence of immunodominant kinesin gene, the number of repeats were highly variable. While *L. chagasi* had 6.5 repeat units Indian strains had only 4–4.5 tandem repeat units.

The strain DD8 kinesin gene showed maximum sequence divergence from non-Indian isolates, as it would be expected. However, even Indian strains had 17–18% sequence divergence. We hypothesize that most important reason for this sequence variation could be the effect of long-term *in vitro* cultivation of its promastigotes. In a study by Nolan and Herman (1985), it was found that *L. donovani* promastigotes that had been subcultured for many years showed decreased infectivity and lack of virulence compared to newly transformed promastigotes. It is also reported that, long-term passaged promastigotes will have lower expression of

particular antigens when compared to short-term passaged promastigotes (Doran and Herman, 1981; Beetham et al., 2003). Moreover, the expression of surface lipophosphoglycan (LPG) and the lipophosphoglycan-associated kinetoplastid membrane protein (KMP)-11 was studied in the strain AG83 of *Leishmania donovani* in axenic culture. The expression of LPG and KMP-11 as well as parasite virulence decreased with the number of *in vitro* subcultures (Mukhopadhyay et al., 1998). Present study also shows similar findings. Our results have special significance because several workers have used DD8 strain in their experiments and in view of present findings results of these studies cannot be translated for currently disease causing strains (Goel et al., 1999; Afrin et al., 2001). Singh et al. (1995a,b) studied genetic heterogeneity among 5 Indian isolates based on kinetoplast and nuclear DNA polymorphisms, in which five isolates could be grouped in to three different types. In another study involving the characterization of Leishmania isolates from India by genomic DNA and kDNA RFLP, it was found that, newer isolates of *L. donovani* were having different patterns thus grouped separately from that of the older isolates from India, e.g. DD8, RMRI (Kapoor et al., 1998). Shamsuzzaman et al. (2000) observed that two WHO reference strain for *L. donovani*, the DD8 strain and HU3 strain showed distinct electrophoretic mobility for the 11 soluble isoenzymes studied. A recent study from our laboratory earlier using *β-tubulin* gene locus as marker clearly showed genetic heterogeneity between the DD8 and KE16 strains (Dey and Singh, 2007).

Interestingly, in terms of antibody titers, inspite of low repeat numbers seroreactivity of recombinant antigens prepared from Indian strains (Ld-rKE16 and Ld-rDD8) was more than *L (d) chagasi* Lc-rK39) which has more tandem repeats (Sivakumar et al., 2006). Further, the study shows that number of tandem repeats was less important than the sequence of expressed antigens. Our two recombinant antigens (Ld-rKE16 & Ld-rDD8) had change in amino acid sequences at position 18, 27, 29, 32 and 38 (Singh and Sivakumar, 2005). The recombinant antigen prepared from DD8 strain showed much poorer sensitivity (Table 3) and specificity (Fig. 4) than the recombinant antigen prepared from KE16 (newer isolate) strain. For this reason we did not commercialize the rDD8 antigen while rKE16 is commercialized successfully.

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